FUNCTIONAL ASPECTS OF THE PERINEURIAL BARRIER IN NORMAL AND PROTEIN-DEPRIVED RATS

Anders Oldfors

Göteborg 1980

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ABSTRACT

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OLDFORS, A. Division of Neuropathology, Department of Pathology, University of Göteborg, S–413 45 Göteborg, Sweden

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The sciatic nerve diffusion barriers to extraneurally applied macromolecules, with special reference to the perineurium have been studied in normal and protein-deprived rats. The protein deprivation was achieved by feeding female rats a protein deficient diet before, during and after pregnancy. After weaning the offspring were fed the same protein-deficient diet. This resulted in considerably reduced body growth and moderately reduced values of albumin and total protein in serum of the offspring. The mean nerve fibre diameter, which was studied in the dorsal tail nerve, was slightly larger in the protein deprived rats at 6 weeks of age. At 15 weeks of age the inverse relation was noted. Motor nerve conduction velocity in the corresponding nerve was not significantly reduced in the protein-deprived rats during the period from 5 weeks to 30 weeks of age. Local extraneural application of the neurotoxic diphtheria toxin to the sciatic nerve resulted in a reversible hind limb paralysis and nerve fibre damage in young and adult protein-deprived and control rats. The protein deprived rats were, however, more seriously affected at 6 and 12 weeks of age. Extraneurally applied horseradish peroxidase (HRP) readily diffused into the endoneurium of sciatic nerves in young but not in adult rats. Penetration of HRP into the endoneurium could also be demonstrated in adult rats by the accumulation of tracer in endoneurial cells, presumably macrophages. Indications of vesicular transport of HRP across the perineurial cells were found. The maturation of the perineurium as a diffusion barrier to HRP was delayed in protein-deprived rats. The passage of ^{125}I-labelled albumin across the perineurium was studied by means of ultrastructural autoradiography. The results suggested a slow passage across the perineurium of the sciatic nerve in adult rats. No obvious differences were found between normal and protein-deprived rats. In view of the demonstration of endoneurial cells with the capacity to endocytose horseradish peroxidase, the occurrence of endoneurial cells with features characteristic of macrophages was studied. Such characteristics included a high activity of acid phosphatase visualized with enzyme histochemistry, a capacity for rapid endocytosis of colloidal carbon and the ultrastructural morphology. Cells fulfilling these criteria accounted for 2 – 4 per cent of the endoneurial cell population.

Conclusions: 1. The perineurium of the sciatic nerve is to some extent permeable to macromolecules in normal adult rats. 2. Penetration of macromolecules across the perineurium is probably mediated by vesicular transport. 3. Cells with characteristic features of macrophages belong to the normal endoneurial cell population. 4. Pre- and postnatal protein deprivation in rats may, to a limited extent, influence peripheral nerve fibre growth during the postnatal development. 5. The motor nerve conduction velocity and nerve fibre diameter are during development correlated to chronological age and not to body size. 6. The maturation of the perineurial diffusion barrier is delayed in protein deprived rats. 7. This may lead to increased vulnerability of the peripheral nerves to extraneurally applied diphtheria toxin.

Key words: rat – peripheral nerve – protein deprivation – perineurium – macrophages – horseradish peroxidase – diphtheria toxin – labelled albumin – autoradiography – nerve fibre diameter – development – nerve conduction velocity.

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Division of Neuropathology
Department of Pathology
University of Göteborg
Göteborg, Sweden

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ABBREVIATIONS

PCM	Protein-calorie malnutrition
LP	Low protein, protein deprived
NP	Normal protein, control
HRP	Horseradish peroxidase
DiT	Diphtheria toxin
MCV	Motor nerve conduction velocity

CONTENTS

INTRODUCTION 5

The peripheral nervous system in protein-calorie malnutrition 5
Tissue barriers of peripheral nerves 6
Perineurium 6
Blood vessels 9

AIMS OF THE INVESTIGATION 9

MATERIAL AND METHODS 10

Animals 10
Protein deprivation 10
Motor nerve conduction velocity and nerve fibre diameter 12
Barrier studies 14
Diphtheria toxin 14
Horseradish peroxidase 15
^{125}I-labelled albumin 17
Macrophages in normal peripheral nerves 18

RESULTS 19

Motor nerve conduction velocity and nerve fibre diameter 19
Barrier studies 20
Diphtheria toxin 20
Horseradish peroxidase 20
^{125}I-labelled albumin 22
Macrophages in normal peripheral nerves 25

DISCUSSION 25

Normal rats 25
Diphtheria toxin 25
^{125}I-labelled albumin 25
Horseradish peroxidase 27
Macrophages in normal peripheral nerves 27
Concluding remarks 28
Protein-deprived rats 28
Motor nerve conduction velocity and nerve fibre diameter 28
Diphtheria toxin 29
Horseradish peroxidase 29
^{125}I-labelled albumin 30
Concluding remarks 30

SUMMARY AND CONCLUSIONS 31

ACKNOWLEDGEMENTS 33

REFERENCES 34

INTRODUCTION

Protein-calorie deficiencies include malnutrition diseases which are secondary to an insufficient calorie supply and/or protein deprivation (Joint FAO/WHO Expert Committee on Nutrition; see WHO 1962). These diseases are major factors in the high morbidity and mortality in underdeveloped areas of the world. The commonly used term for this group of diseases is protein-calorie malnutrition (PCM) (*cf.* Olson 1975). During the last two decades much interest has been focussed on the effects of malnutrition on brain development (for reviews see: Dodge *et al.* 1975; Wurtman and Wurtman 1977). This field of research has several important aspects. Some of these include questions concerning the distribution and quality of lesions in the nervous system and their significance for the physical and mental development, vulnerable age periods and the relative importance of different nutritional factors.

The peripheral nervous system in protein-calorie malnutrition

Studies on the peripheral nervous system have revealed that a marked reduction of motor nerve conduction velocity (MCV) may occur as one manifestation of severe PCM in children (Osuntokun 1971, Sachdev *et al.* 1971, Engsner and Woldemariam 1974, Singh *et al.* 1976). The reason for this reduction of MCV, which is reversible after nutritional rehabilitation, has not been clarified. Results from quantitative morphometric studies on the peripheral nerves in children suffering from PCM suggest an impaired development of medium and large myelinated nerve fibres (Dastur *et al.* 1977). These findings are in agreement with the results reported by Sima (1974 a) from studies on peripheral nerves in undernourished rats. Studies on conduction velocity of the fastest fibres in the dorsal roots of these rats revealed moderately reduced values (Sima and Jankowska 1975). The reduction was proportional to the reduction of diameter of the corresponding fibres. Early nutritional deprivation has been shown to cause decreased axonal growth as well as impaired myelin deposition in peripheral nerves of the rat (Clos and Legrand 1970, Hedley-Whyte and Meuser 1971, Hedley-Whyte 1973, Sima 1974 b). Roy *et al.* (1972) recorded markedly reduced MCV in young monkeys fed a protein-deficient diet. They correlated this finding to ultrastructural alterations in the nerve fibres. Studies on teased nerve fibres from pre- and postnatally protein-deprived rats have revealed no signs of peripheral neuropathy (Oldfors and Nordborg 1977, Nordborg 1978). After severe postnatal undernutrition in rats Haltia (1970) found a decreased neuronal dry mass and RNA content in spinal anterior horn neurons. However, in pre- and postnatally undernourished rats whose weights were less severely reduced the RNA content of spinal anterior horn neurons was not significantly reduced (Sourander *et al.* 1976). Moderate pre- and postnatal undernutrition in rats did not significantly affect the spinal ganglion nerve cell size (Oldfors and Sourander 1977).

The interaction of malnutrition and infection is well known from human pathology. Among other important factors, impaired tissue barriers have been held to contribute to the diminished resistance to infections in malnourished children (*cf.* Scrimshaw 1968). The peripheral nerve fibres are enveloped by tissue barriers consisting of the perineurium and the endoneurial vessel walls. It has been assumed that these barriers protect the nerve fibres from toxic or infectious agents in the surrounding tissues (Thomas and Olsson 1975, Kristensson and Olsson 1976). Furthermore, this barrier function of the perineurium has been claimed to be irreversibly damaged in undernourished rats (Sima and Sourander 1973, 1974).

Tissue barriers of the peripheral nerves

The peripheral nerves are composed of one or more fascicles consisting of a variable number of individual nerve fibres. According to the generally accepted nomenclature, the nerves are divided into three compartments (Key and Retzius 1876) (Fig. 1): the endoneurium which represents the mesenchymal structures and extracellular space within the fascicle, the perineurium, which constitutes a specialized sheath around each fascicle, and the epineurium, which is the extrafascicular connective tissue. Together with the endoneurial vessel walls, the perineurium forms a barrier system separating the nerve fibres from the systemic extracellular fluids.

Perineurium

Morphology: The perineurium is a cell sheath which appears as an extension from the pia-arachnoid mater of the central nervous system (CNS) covering the spinal and cranial nerve fascicles to their peripheral terminations (Shantaverappa and Bourne 1964). However, direct continuity between the root sheaths and the perineurium is lacking (McCabe and Low 1969). The number of the cell layers in the perineurium decreases in the periphery as the nerve fascicles decrease in size. At the motor end plate the last sleeve ends shortly before the termination of the nerve (Burkel 1966, 1970, Saito and Zacks 1969). In various sensory nerve endings the perineurium continues

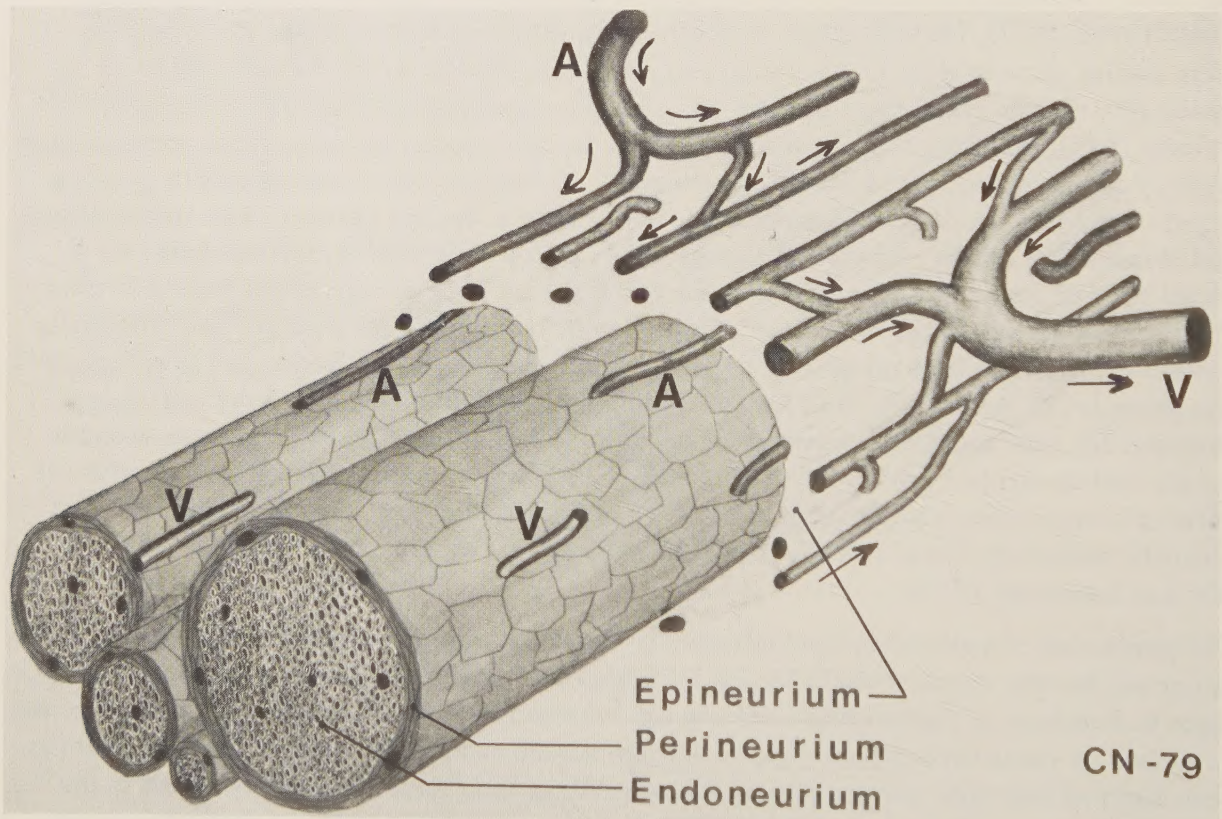


Fig. 1. Schematic illustration of a large peripheral nerve trunk. The perineurium surrounds each fascicle. The connective tissue outside the perineurium constitutes the epineurium. The endoneurium is the extracellular space and the mesenchymal elements inside the perineurium.

to form the capsule of the nerve ending, *e.g.* the lamellae of Pacinian corpuscles (Shantaverappa and Bourne 1963, Polacek and Halata 1965). The perineurial cell layers are composed of extremely flattened cells, sometimes measuring less than 100 nm in cross section (Röhlich and Knoop 1961). The perineurial cells of all layers exhibit large numbers of micropinocytotic vesicles measuring 50 – 60 nm (Röhlich and Knoop 1961, Cravioto 1966, Akert *et al.* 1976). The cells of each layer are fitted together by overlapping and dovetailing. "Closed cell contacts" or "tight junctions" are a constant finding at these sites (Thomas 1963). By means of the freeze-etching technique, extensive zonulae occludentes have been demonstrated at these cell contacts (Reale *et al.* 1975, Akert *et al.* 1976). Each cell layer is usually covered by a basement membrane on both sides. In the extracellular spaces between the cell layers bundles of collagen fibres are present.

Enzyme histochemistry: The results of the enzyme histochemical studies made on peripheral nerves have been reviewed by Thomas (1977). Although there are inter-species variations, the perineurium is generally held to show high activity of different dephosphorylating and oxidative enzymes. Adenosine triphosphatase has been demonstrated electron-microscopically in micropinocytotic vesicles and in the basement membranes of the perineurial cells (Novikoff *et al.* 1966, Schlaepfer *et al.* 1969).

Ontogenesis: The embryological origin of the perineurium has not been established. Based on the assumption that the perineurium constitutes a direct continuation of the root sheaths, which possibly are of neuroectodermal origin, Shantaverappa and Bourne (1963) considered the perineurium to be of neuroectodermal origin. During development (Gamble and Breathnach 1965) or regeneration (Thomas and Jones 1967) the perineurium is derived from cells with morphological similarities to fibroblasts. The perineurial cells also exhibit morphological (Ross and Reith 1969) and enzyme histochemical (Novikoff 1967) similarities to smooth muscle cells.

Barrier and transport properties: The perineurium of large nerve trunks in vertebrates acts as a diffusion barrier to various substances, ranging from electrolytes (*cf.* Martin 1964) to large molecular substances such as ferritin (mol. weight: 465 000) (Waggener *et al.* 1965, Hall and Williams 1971). Several studies have dealt with the barrier function of the perineurium to different macromolecular substances. Albumin (mol. weight: 69 000) labelled with Evans blue (EBA) did not penetrate from the epineurium into the endoneurium of rats (Olsson 1966 a, Kristensson and Olsson 1971, Sima and Sourander 1973), mice (Olsson and Reese 1971, Kristensson and Olsson 1971) or man (Söderfeldt *et al.* 1973). Diphtheria toxin (mol. weight: 62 000), which is known to cause segmental demyelination after endoneurial injection, did not affect the nerve fibres within 14 days after epineurial administration in adult rats, while demyelination occurred in weanling rats within a few days (Boettge 1975). This lack of demyelination in adult rats, was interpreted as being due to the efficient diffusion barrier properties of the perineurium. Horseradish peroxidase (HRP; mol. weight 40 000) which was locally applied around the sciatic nerves or injected into the nerves of mice (Olsson and Reese 1969, 1971) and rats (Klemm 1970, Böck and Hanak 1971) penetrated only to the intermediate layers of the perineurium. Klemm (1970) stated that this lack of passage across the intermediate layers was due to the insignificant vesicular transport in these layers compared to the outer and inner layers. Olsson and Reese (1971) suggested that the penetration stopped at the completely concentric perineurial cell layers and expressed doubts as to whether vesicular transport over perineurial cells occurred at all. Later tracer studies support the concept of the perineurium as an efficient barrier to HRP in adult rats (Sima and Sourander 1974, Kristensson and Olsson 1976). Arvidson (1977), in a study on sciatic nerve of mice noted, however, that

endoneurial cells, presumably macrophages, accumulated HRP after local extraneural application of the tracer. It was postulated that this could be explained by an absorption of tracer in local epineurial blood vessels and a subsequent passage of tracer into the endoneurium *via* the endoneurial blood vessels. This hypothesis was partly based on the finding that endoneurial blood vessels were to some extent permeable to HRP.

The barrier function of the perineurium has been correlated to the morphology of the perineurium, with multiple "tight junctions" between the cells. In young mice and rats, in which such extensive cell contacts are lacking the perineurium does not act as a diffusion barrier to macromolecules (Kristensson and Olsson 1971, Sima and Sourander 1973, 1974). The perineurial diffusion barrier is very resistant to ischaemia (Lundborg *et al.* 1973) and is not altered after local administration of histamine, serotonin and bradykinin (Söderfeldt 1974). The permeability of the perineurium may be drastically increased by crush injury (Olsson and Kristensson 1973) and after exposure to hyperosmolar solutions (Kristensson and Olsson 1976). Weerasuriga *et al.* (1979) showed that stretching of the perineurium increased the permeability *in vitro*. Sima and Sourander (1973, 1974) reported that the development of the perineurium as a diffusion barrier was impaired after pre- and postnatal undernutrition in rats. This was manifested as a remaining leakage of extraneurally applied protein tracers across the perineurium in adult undernourished rats. This was also seen after nutritional rehabilitation. The function of the perineurium as an efficient barrier was questioned by Lorente de Nó (1950) and more recently by Kirkpatrick and Reid (1976). According to Krishnan and Singer (1973), HRP penetrated the entire perineurium by means of vesicular transport after repeated and prolonged application of HRP around peripheral nerves in the newt.

The above quoted tracer studies have dealt with large nerve trunks. After systemic administration of HRP (Böck and Hanak 1971) or local injections of Cytochrome C (mol. weight: 12 000) (Malmgren and Brink 1975) the tracers were identified in small nerve branches of the tongue in rats. These small nerves lacked endoneurial vessels; it was postulated that the tracers had diffused from the interstitium into the nerves in a retrograde direction *via* the nerve endings.

According to Shantha and Bourne (1968), the enzyme activities and barrier functions of the perineurium provide evidence that the perineurium acts as a metabolically active diffusion barrier in the peripheral nerves. Krnjević (1955) proposed that, since endoneurial fluid has a markedly higher osmolarity than plasma, the necessary continuous removal of water is performed by the perineurium. Van Lis and Jennekens (1977) discussed the removal of extravasated plasma protein from the endoneurium in normal and pathological conditions. Based on the relatively high endoneurial protein content they suggested a slow removal process, possibly *via* endoneurial blood vessels or the perineurium. Allt (1972) and de la Motte *et al.* (1975) proposed that the perineurium plays an important role in the disposal of leaked protein. This was based on the finding that endoneurially extravasated proteins in different pathological conditions could be traced between all the perineurial laminae as well as in macropinocytotic vesicles of the perineurial cells.

Additional functions of the perineurium: In addition to the mechanical protection of the nerve fibres and the barrier and transport properties described above, it has also been proposed that the perineurium may have contractile capacity. Ross and Reith (1969) described perineurial cell filaments comparable to the myofilaments seen in smooth muscle cells, and Becker and Murphy (1972) have demonstrated smooth muscle actomyocin in the perineurial cells with immunohistochemical methods. The slow proximo-distal flow of fluid in the endoneurium described by Mellick

and Cavanagh (1967) is of interest in this context.

Blood vessels

Regional arteries enter the epineurium segmentally, where they ramify in ascending and descending branches (Adams 1942). Precapillary vessels penetrate the perineurium (Sunderland 1945). The blood vessels inside the perineurium form a capillary plexus (Waksman 1961), and the vessels leaving the endoneurium are capillaries or small venules. The number of venules within the nerve fascicles varies between different species (Waksman 1961). The blood vessels penetrate the perineurium very obliquely and are surrounded by a perineurial sleeve for some distance in the endoneurium (Gunderson and Low 1968). The epineurial vessels are generally more permeable to macromolecules compared to endoneurial vessels, which constitute the so-called blood-nerve barrier, but the permeability of endoneurial vessels varies between different species (Waksman 1961, Olsson 1971). Small nerve branches lack endoneurial vessels (Böck and Hanak 1971, Malmgren and Brink 1975). The permeability of endoneurial vessels of the peripheral nerves increases after administration of 5-hydroxytryptamine, histamine and Compound 48/80 (Olsson 1966 b). While lymphatic vessels are present in the epineurium, no lymphatic vessels have been demonstrated in the endoneurium (*cf.* Sunderland 1968).

AIMS OF THE INVESTIGATION

The previous reports that the perineurium under normal conditions is impermeable to macromolecules in adult rats and that the development of the barrier function of the perineurium is impaired in pre- and postnatally undernourished rats have given rise to two important questions: firstly, if the perineurial barrier is inefficient in undernourished rats, what are the pathways of leakage across the perineurium? Secondly, are the sciatic nerves of undernourished rats more vulnerable to neurotoxic macromolecular substances because of an inefficient perineurial barrier? In order to elucidate these questions, the perineurial barrier was studied in normal and protein deprived rats at various ages during development. These studies were performed using a neurotoxic macromolecular substance and macromolecular tracers applied extraneurally.

In order to obtain information on the effect of the protein deprivation on the development of peripheral nerve fibres, morphometric and functional studies were performed at various ages during the postnatal development.

During the tracer experiments, accumulation of tracer was seen in endoneurial cells, presumably macrophages. Macrophages are known to contribute to the protection of the organism. A review of the pertinent literature has not revealed any previous ultrastructural studies on the occurrence and morphology of endoneurial macrophages in normal peripheral nerves. Against this background, it seemed to be of interest to study the occurrence of endoneurial cells with features characteristic of macrophages.

MATERIAL AND METHODS

Animals

Various species have been used in previous experimental studies on the effects of early malnutrition on the nervous system including rats, mice, pigs, dogs and monkeys (*cf.* Shoemaker and Bloom 1977). Well-known inbred strains of albino rats *e.g.* Sprague-Dawley or Wistar strains are however most commonly used. The difficulties in drawing direct parallels to human malnutrition must, however, be borne in mind (Widdowson 1968). Dobbing (1973) stressed the importance of comparing stages and not ages. For instance, the brain growth spurt of the rat begins at about birth, whereas in man it starts at about mid gestation (Dobbing 1973). This is of importance when the vulnerable periods of brain development are considered (Dobbing and Sands 1971). In this study Sprague Dawley rats have been used. The animals were kept in plastic cages in a room which was illuminated from 6 a.m. to 6 p.m. and had a temperature of 22°C and a relative humidity of 60 per cent.

Protein deprivation

Experimentally induced PCM for studies on the nervous system in rats may be achieved in several ways, as follows:

1. Undernutrition by dietary means only after weaning, either by reduction of the amount of food (*e.g.* Dobbing and Widdowson 1965, Yu *et al.* 1974) or by reduction of the protein content of the food (*e.g.* Hatai 1904, Enwonwu and Worthington 1975).
2. Undernutrition by separation of the offspring from lactating mothers for various lengths of time, implying a reduced suction time (*e.g.* Sugita 1918, Haltia 1970, Salas *et al.* 1974).
3. Undernutrition by inducing abnormally large litter sizes (*e.g.* Sugita 1918, Winnick and Noble 1966, Dobbing *et al.* 1971).
4. Undernutrition by reduction of the food given to lactating mothers (*e.g.* Sima 1974 a, b, Lewis *et al.* 1975, Oldfors and Sourander 1977).
5. Undernutrition by giving the lactating mothers and their offspring a protein deficient diet (*e.g.* Shoemaker and Wurtman 1973, Stewart *et al.* 1974, Oldfors and Nordborg 1977, Nordborg 1978, Morgane *et al.* 1978, Conradi *et al.* 1979 a, b).

In this study a PCM model, which involves protein deprivation, was used. The protein deprivation was achieved by feeding female unmated rats *ad libitum* a diet containing 7 weight per cent fish protein from two weeks before conception and onward. After weaning, the offspring were fed the same diet. Control animals were fed a balanced diet containing 14 weight per cent fish protein. The two different diets were approximately isocaloric and contained vitamins in quantities well above normal requirements (McCoy 1963). The composition of the diets is described in Table I. The diets were prepared in our laboratory and given to the animals in the form of pellets. All litters were adjusted to 7 - 8 animals. Henceforth in the text the protein deprived rats will usually be referred to as LP (low protein) rats and the controls as NP (normal protein) rats.

The effects of the protein deprivation on body weight growth and serum content of albumin and total protein are summarized in Fig. 2 and Table II. The protein deprived rats showed a considerably reduced and delayed body growth in terms of both weight and length, including the tail. The

Table I. Composition of the diets.

	NP	LP
Weight per cent protein[1]	14	7
Constituents in 25 g of diet, g		
Protein (SP-protein, Swedish Protein AB)	4.460	2.230
Wheat starch	15.500	17.700
Sucrose	1.300	1.300
Hydrogenated lard	1.950	1.950
Sunflower oil	415	415
Linseed oil	150	150
Cellulose	900	900
Salt mixture[2]	1.000	1.000
Choline chloride	50	50
Vitamine mixture[3]	37	37

1. Calculated on the basis of crude protein content: 80 per cent fish protein according to manufacturer (Swedish Protein AB).
2. Salts in 25 kg of diet: $MnSO_4 \cdot 7H_2O$ 4.0 g; NaCl 139.3 g; KH_2PO_4 389.0 g; $MgSO_4$ 57.3 g; $CaCO_3$ 381.4 g; $FeSO_4 \cdot H_2O$ 27.0 g; KI 790 mg; $ZnSO_4 \cdot 7H_2O$ 548 mg; $CuSO_4 \cdot 5H_2O$ 477 mg; $CoCl_2 \cdot 6H_2O$ 23 mg; $KAl(SO_4)_2 \cdot 12H_2O$ 87.95 mg; NaF 276.3 mg; $NaAsO_2$ 8.67 mg; $Na_2B_4O_7 \cdot 10H_2O$ 22.03 mg; $Na_2MoO_4 \cdot 2H_2O$ 3.13 mg.
3. Vitamines in 25 kf of diet: Retinol 300.000 I.U.; Thiamin 1250 mg; Riboflavin 500 mg; Panthotenic acid 2500 mg; Nicotinamide 5000 mg; P-aminobenzoic acid 2500 mg; Naphtoquinone 250 mg; Biotin 25 mg; Folic acid 125 mg; Cyanocobalamin 1.25 mg; Myoinosit 25 g; DL-o-tocopherol 12500 mg; Calciferol 105.000 I.U.; Pyridoxine 500 mg.

Table II. Serum content of albumin and total protein in normal (NP) and protein-deprived (LP) rats. Standard deviation within parenthesis.

			Albumin g/l	Protein g/l
LP	6 weeks	n = 5	24 (± 1.2)	42 (± 2.9)
NP	6 weeks	n = 9	31 (± 1.4)	53 (± 2.5)
LP	15 weeks	n = 9	24 (± 1.9)	47 (± 2.4)
NP	15 weeks	n = 9	29 (± 1.9)	61 (± 3.7)

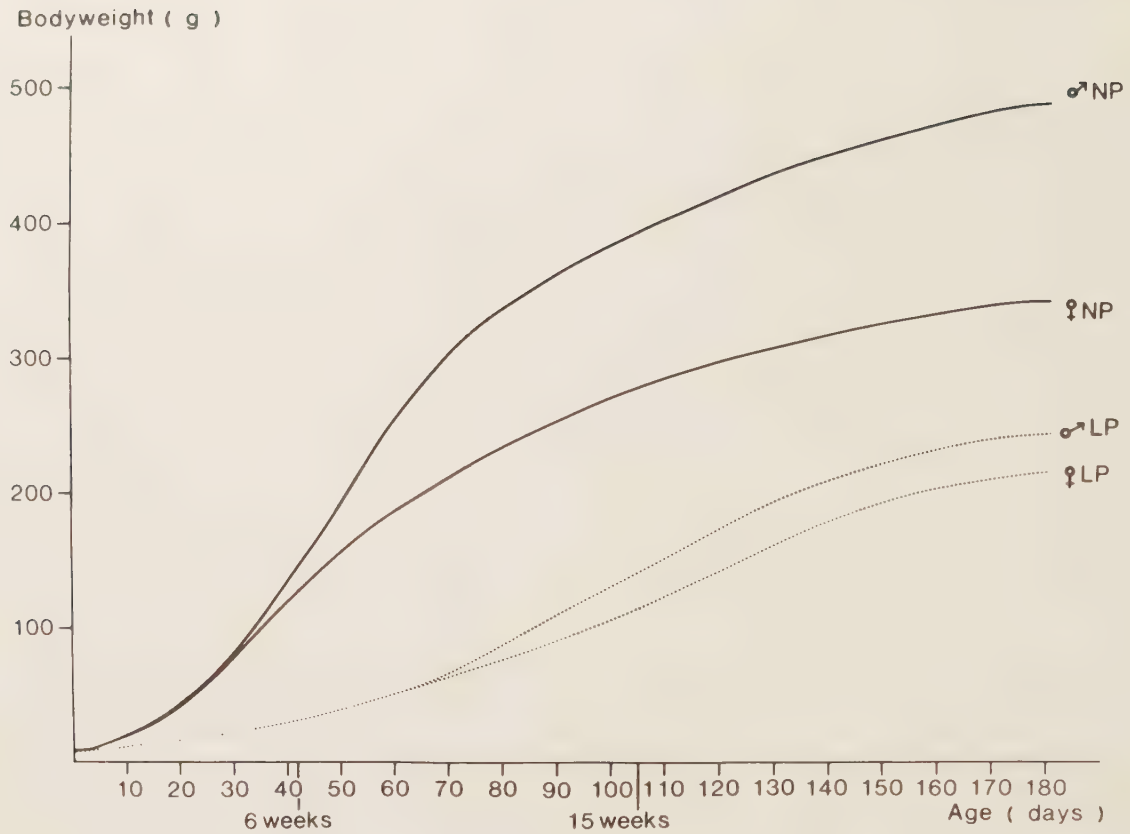


Fig. 2. Body weight of protein-deprived (LP) and normal (NP) rats at different ages during the postnatal development. The growth of the LP rats is decreased and the growth spurt is delayed compared to the NP rats.

serum content of albumin and total protein was moderately reduced at 6 and 15 weeks of age. Although the protein deprived rats showed an increased mortality during the weaning period, the majority survived and appeared healthy. Extreme protein deprivation in rats has been described by Edozien (1968). However, his model of PCM entailed protein deprivation induced after weaning.

Motor nerve conduction velocity and nerve fibre diameter (study I)

In studies on motor nerve conduction velocity and nerve fibre diameters in rat peripheral nerve the use of the tail offers certain advantages. The segmental muscles are small which facilitates the identification of electro-myograms of single motor units. The nerves innervating the distal segmental muscles have a length which makes it possible to stimulate at different levels and thereby determine the nerve conduction velocity. Furthermore, these nerves consist of a moderate number of myelinated nerve fibres, which makes it possible to include the whole population in calibre spectra.

Anatomy: A detailed description of the nerves and muscles in the tail of the rat has been given by Steg (1964). The tail of the rat consists of 27 vertebrae. Three pairs of short tendons of segmental muscles which emerge from the next proximal segment, insert in the proximal end of each verte-

bra. The tail is innervated from six pairs of dorsal and ventral coccygeal roots. The dorsal segmental muscles and the skin at the dorsal aspect of the tail are innervated by branches from two dorsal longitudinal nerve trunks in the tail.

Motor nerve conduction velocity (MCV)

The rats were anaesthetized with urethane (100 mg/kg) or levomepromazin (2.5 mg/kg; Nozinan®, Leo). The tail nerve trunks were blocked in the proximal part of the tail by using lidocaine (10 mg/ml) with nephrine (5μg/ml) (Xylocain®adrenalin, Astra). Stimulating electrodes were inserted close to the left dorsal tail nerve after incisions in the skin at four different levels in the proximal part of the tail. Electromyograms of single motor units were recorded from dorsal segmental muscles at a level corresponding to the 15th to 16th caudal vertebrae. The recording electrodes were inserted in the muscles which were exposed through skin incisions and covered with liquid paraffin. A reference electrode was inserted into non-contractile tissue close to the muscle. The electromyograms of the motor units were displayed on an oscilloscope screen. When the identical all-or-none electromyogram appeared on the oscilloscope screen after stimulation at two or more different levels the MCV could be determined. The conduction velocity of several fibres in each rat was in this way calculated. The temperature of the tail was measured during the recordings by means of a thermocouple. As the temperature may influence nerve conduction velocity (Paintal 1965), a separate series of experiments were undertaken to determine the influence of temperature differences. The temperature dependence of the nerve conduction velocity was found to be close to 1.0 m/s/° C. All values of MCV were corrected by this factor to values corresponding to 32° C.

The study included 11 LP and 11 NP rats at ages ranging from 5 – 35 weeks (LP rats) to 3 – 30 weeks (NP rats). The MCV was determined for 127 nerve fibres of the NP rats and 103 nerve fibres of the LP rats.

Morphometrics

The determination of nerve fibre calibre spectra included one of the dorsal tail nerves at a level corresponding to the 12th – 14th vertebrae. At this level the nerves consisted of about 300 myelinated fibres and it was thus possible to include the whole population. These myelinated nerve fibres represent, however, a mixture of afferent and efferent fibres. For this reason, calibre spectra were also determined on ventral spinal coccygeal and sacral roots. These roots consist mainly of efferent fibres, including the muscle efferents on which MCV was assessed more peripherally in the tail. In order to get a sufficient number of animals in each group for statistical comparison the study was limited to two age-groups: rats aged 6 weeks – 8 LP and 9 NP rats; rats aged 15 weeks – 7 LP and 8 NP rats.

The animals were perfused with a buffered solution of 2.5 % glutaraldehyde and 2.5 % paraformaldehyde in 0.1 M sodium phosphate buffer. The specimens were dissected out, rinsed, postfixed in OsO_4 and embedded in Epon 812. One micron thick cross sections of the nerves and roots were cut on an LKB ultrotome and stained according to the method of Richardson *et al.* (1960). This method of fixation and embedding results in good structural preservation of the nerve fibres and little shrinkage (Sima 1974 a). Micrographs of the sections were reproduced on paper copies at a final magnification of x 770. The diameter of all myelinated nerve fibres was measured by means of a particle size analyzer (Zeiss TGZ 3). The method used represents a modification of that described by Romero and Skoglund (1964). In this study the diameter of the circumscribed circle

of each fibre was determined. In this way calibre spectra from one dorsal tail nerve and several ventral nerve roots from each rat were obtained. Only male rats were included.

Barrier studies (papers II, III and IV)

Choice of methods: As already mentioned (see p. 7), previous studies have shown that various macromolecular substances do not appear in the endoneurium after epineurial application to the sciatic nerves in adult rats. Among these substances were albumin, horseradish peroxidase and diphtheria toxin. It was also found that the function of the perineurial barrier was impaired in undernourished rats (see p. 8). In this study the sciatic nerve barriers to these three macromolecular substances were examined after epineurial application in normal and protein deprived rats. Diphtheria toxin (DiT) causes segmental demyelination with subsequent paralysis of the hind limb after endoneurial injections in rat sciatic nerve, the response being proportional to the dose injected (see below under "Diphtheria toxin"). It was therefore considered possible to obtain a rough estimate of the efficiency of the sciatic nerve barriers by studying the effect of extraneurally applied DiT. Horseradish peroxidase was used as a tracer in order to be able to study the possible routes of leakage across the perineurium and the subsequent distribution of a macromolecular tracer within the endoneurium. It was also used to obtain information on large differences in the perineurial barrier function in animals of different ages and nutritional state. Albumin labelled with ^{125}I and ultrastructural autoradiography were used to study the permeability of the perineurium in quantitative terms.

Diphtheria toxin (DiT)

DiT is a protein (mol. weight 62 000) produced by Corynebacterium Diphtheriae and acts as an exotoxin. When DiT is administered systematically one important effect is peripheral neuropathy by demyelination (Waksman *et al.* 1957, Webster *et al.* 1961, McDonald 1963, Weller 1965). According to Pleasure *et al.* (1973), this effect is due to inhibition of the synthesis of basic protein and proteolipids in the Schwann cells. The toxin is built up of two polypeptides, one which is necessary for the binding to the cell surface and one which exerts the synthesis inhibiting effect inside the cell (*cf.* Collier 1975). Variations in distribution of nerve lesions between different species have been proposed to be partly due to differences in the blood-nerve-barrier (Waksman 1961). The effects of DiT have also been studied by local endoneurial injections of the toxin in young and adult rats (Agarvall and Pryce 1959, Jacobs 1967, Allt 1969, Allt and Cavanagh 1969) and by epineurial injections in immature rats (Weller and Nester 1972, Boettge 1975). The neuropathic effect and latency period after endoneurial toxin administration are dose-dependent (Cavanagh and Jacobs 1964). The latency time is also age-dependent. Young rats developed nerve fibre damage within a considerably shorter time after the endoneurial toxin administration than adult rats (Jacobs 1967). Although the dominating effect is segmental demyelination, axonal degeneration has also been described (Bradley and Jennekens 1971, Brown and Gilbert 1974). However, the functional disturbance caused by the demyelination is reversible and when remyelination has started the restoration is a relatively rapid process (Jacobs 1967, Mellick *et al.* 1975). The DiT used in this study was obtained from the National Bacteriological Laboratory, Stockholm, Sweden. The stock solution contained 500 Lf DiT per ml. (For definition of the Lf unit, see: Glenny and Okell 1924). 10 µl of the toxin was locally applied to the right sciatic nerve of rats of different ages. The sciatic nerves of rats of the youngest age-group (*i.e.* rats aged 3 weeks) were exposed to 0.5 Lf of toxin while in the other age-groups (*i.e.* rats aged 6, 12 and 26 weeks) the sciatic nerves

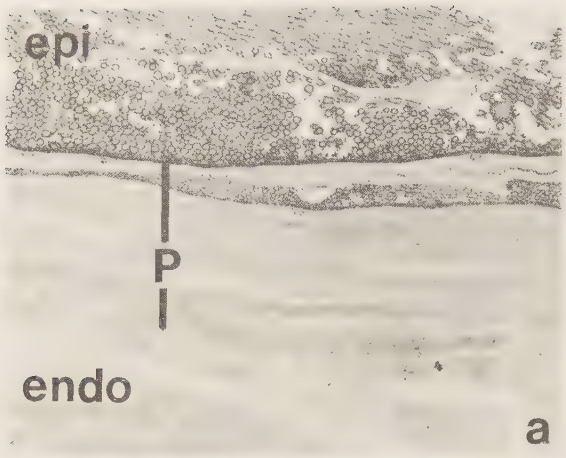
were exposed to 5 Lf of toxin. The effect of the toxin was estimated by studying the degree of hind limb paralysis every day throughout the period of functional disability. This was done according to a schedule described by Weller and Nester (1972). Groups of animals with different degrees of functional disturbance were killed to study whether the functional disturbance was roughly correlated to the nerve fibre damage. After immersion fixation in a buffered solution of glutaraldehyde and paraformaldehyde, the sciatic nerves, at the level from the sciatic notch to the distal femur, were prepared according to the OTAN-method (Adams 1965). The nerves were macerated in 60 % glycerine and teased into single nerve fibres or small bundles of fibres. Two hundred nerve fibres from each nerve were inspected light-microscopically and the proportion of severely damaged nerve fibres was estimated.

Horseradish peroxidase (HRP)

HRP is a protein with a molecular weight of approximately 40 000. The presence of HRP can be visualized both light- and electron-microscopically after reaction with 3,3′-diamino-benzidine (DAB) (Graham and Karnovsky 1966). In the presence of HRP and hydrogen peroxide, DAB undergoes oxidative polymerization to form an osmiophilic end product (Seligman *et al.* 1968).

The advantages of using HRP as a tracer are the high resolution of tracer activity, which makes it possible to trace the HRP in subcellular structures, and the sensitivity of the method (Graham and Karnovsky 1966). However, the results from tracer studies must be interpreted with caution both when a positive reaction is obtained and when such is lacking. Endogenous peroxidase activity is found in several different types of cells mainly those of haematogenous origin, for example monocytes and macrophages (Nichols and Bainton 1975), but also in other cells *e.g.* cells of the CNS (Keefer and Christ 1976, Wong-Riley 1976). It must also be stressed that the HRP method is not strictly quantitative. This is important when interpreting the results from barrier studies. The possibility of detecting the HRP activity with the DAB method as used in the present study depends on the concentration of the tracer (see Fig. 3). Another factor of importance is the slow penetration of DAB into the specimens. The incubated specimens must therefore be as thin as possible. The presence of large amounts of HRP may also negatively influence the sensitivity of the method in adjacent parts of the specimens (own observations). Lack of reaction product must therefore not be interpreted as necessarily being due to the absence of peroxidase. Another disadvantage of the HRP method in studies on vascular permeability is the sometimes occurring allergic reactions with mast cell degranulation (Cotran and Karnovsky 1967).

In this study (papers III and IV) the HRP (Sigma, type IV) was locally applied around the sciatic nerves. Ten microlitres of a solution containing 3 mg of HRP (paper III) or 3 mg/ml (paper IV) was dripped on to the surface of the nerves. During this procedure the animals were anaesthetized with ether. After various latency times *i.e.* 2 hours, 1 day and 3 days (paper III) and 10 min., 30 min. and 2 hours (paper IV) the animals were killed by decapitation and the nerves were immersion fixed *in situ* with a buffered solution containing glutaraldehyde and paraformaldehyde. After rinsing, thin slices (approx. 300 μm) of the specimens were cut by means of a tissue chopper and incubated in the DAB medium. In study III this contained 5 mg DAB dissolved in 10 ml of 0.05 M Tris buffer at pH 7.5 (Graham and Karnovsky 1966). After 12 hours preincubation, 0.1 ml 1 % H_2O_2 was added to the medium, for an additional 4 hours. In study IV the specimens were incubated in a medium (Malmgren and Olsson 1977) containing 20 mg of DAB dissolved in 10 ml 0.1 M sodium cacodylate-HCl buffer at pH 5.1 for 30 min. 0.1 ml of 1 % H_2O_2 was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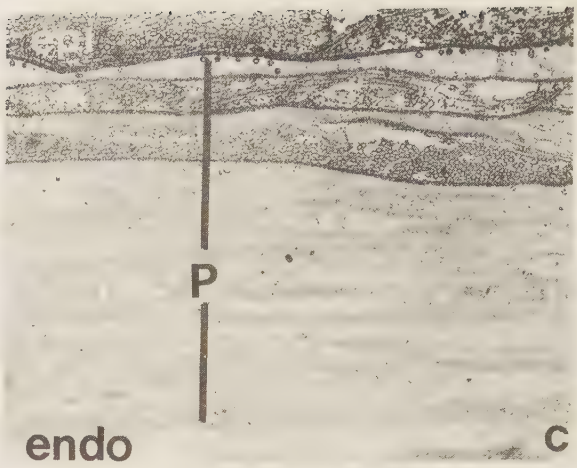
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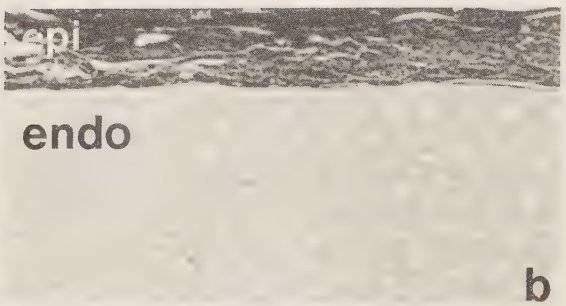
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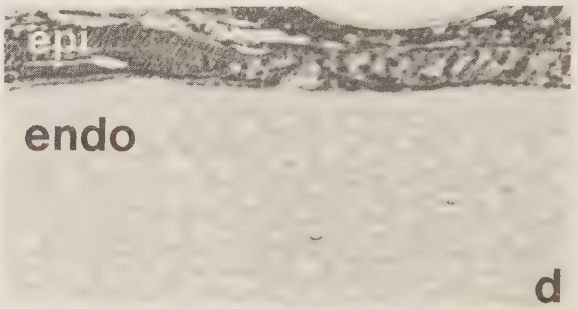
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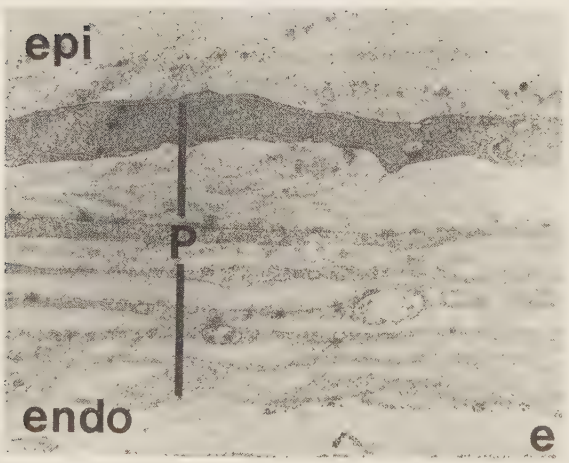
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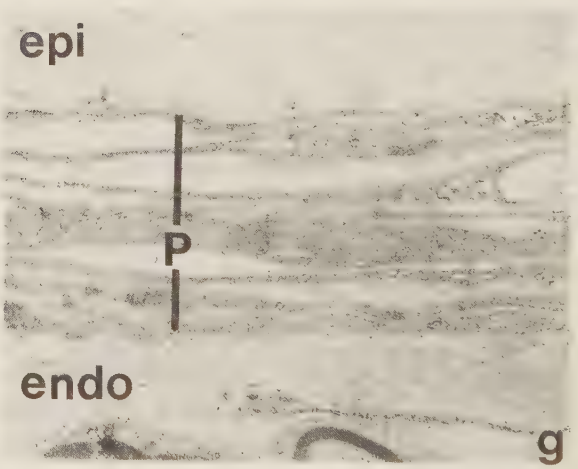
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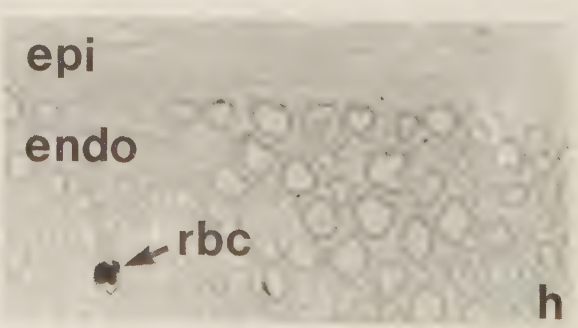
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then added to the medium (pH 5.1), for an additional 60 min. After the incubation, the specimens were postfixed in 1 % OsO_4 and embedded in Epon 812. One micron thick sections for light microscopy or ultrathin sections (50 – 60 nm) for electron microscopy were cut on an LKB ultrotome. The specimens were usually not poststained. Study III included LP and NP rats aged 4, 6, 12 and 26 weeks. In study IV only NP rats aged 12 weeks were included.

^{125}I-labelled albumin

In contrast to HRP, ^{125}I-labelled albumin may serve as a tracer for quantitative estimations in autoradiographic studies on the relative amounts of tracer in different compartments. This is made possible by the fact that the number of developed silver grains in the emulsion covering the specimens is proportional to the concentration of radioactivity in the specimens. On the other hand, the resolution is not as good as with the HRP method, although electron-microscopical autoradiography provides higher autoradiographic resolution than the light-microscopical technique. The limited resolution is due to several factors, the most important being the spread of radiation from the source to the site of image formation. This is dependent on the thickness of the specimen and the type and thickness of the overlying emulsion (*cf.* Salpeter *et al.* 1972). This factor, and the background activity, must be considered when analyzing the autoradiogram (Salpeter and McHenry 1973). In this study ^{125}I-labelled bovine serum albumin (NEX 076, NEN Chemicals) (mol. weight 69 000) was used. The albumin was dissolved in phosphate buffer, pH 7.5, with a protein concentration of 0.7 mg/ml and a specific activity of 2.4 mCi/mg according to the manufacturer. The free ^{125}I-content was less than 3 % according to the manufacturer. The study was limited to one age-group (LP and NP rats aged 12 weeks) because of the time-consuming

Fig. 3. The effect of concentration of HRP upon the visibility of the reaction product.

10 µl of a solution containing HRP (Sigma Type VI) of varying concentration was applied to sciatic nerves of rats 5 minutes before fixation by perfusion with glutaraldehyde and paraformaldehyde. 300 µm thick cross sections from different levels of the nerves were incubated in DAB according to the method of Malmgren and Olsson (1977). After fixation in OsO_4, the specimens were embedded in Epon 812. One micron thick sections were cut for light microscopy, and ultrathin sections were cut for electron microscopy. The figures illustrate the peri-, endo- and epineurium in the areas where the highest activity of peroxidase was found.

a and b 3 mg HRP/ml
c " d 0.3 " "
e " f 0.03 " "
g " h 0 " "

a, c, e and g: electron microscopy x 11.000
b, d, f and h: light microscopy x 480

C o m m e n t: Using 3 or 0.3 mg HRP/ml, high activity of peroxidase (dark reaction product) can be identified in the epineurium and outer part of the perineurium both electron- and light-microscopically. However, when 0.03 mg/ml was used no reaction product could be unequivocally identified in the corresponding area.

epi = epineurium; endo = endoneurium; p = perineurium; rbc = red blood corpuscle.

grain counting. As the time from application of the tracer to possible penetration into the nerve was not known, observations were made at three different points of time – 10, 30 and 120 minutes. Therefore, only a limited number of animals were included in each group. The specimens which were technically successful and could be used for analysis represented at 10 min. 2 LP and 1 NP rats, at 30 min. 2 LP and 1 NP rats and at 120 min. 2 LP and 2 NP rats. Ten microlitres of the ^{125}I-labelled albumin was locally applied to the sciatic nerve. After different latency times the nerves were fixed by immersion in 2 % glutaraldehyde and 2.5 % paraformaldehyde in 0.05 M sodium cacodylate buffer. After rinsing in buffer, specimens including the proximal part of the sciatic nerve just distal to the sciatic notch were dissected out. The specimens were postfixed in OsO_4 and embedded in Epon 812. Ultrathin sections with a pale golden to silver interference colour corresponding to a specimen thickness of 800 – 900 Å were picked up on Formvar-coated copper grids and stained with uranyl acetate and lead citrate. The specimens were coated with a layer of carbon by evaporation, and the emulsion, Ilford L4, was applied with a wire loop. The preparations were stored at + 4°C in the presence of desiccating agent for 4 weeks. They were developed with Kodak FD 19B for 2 min. and fixed for the same time in Kodak F 24 fixer.

Autoradiograms were obtained from, usually, three or four grids, representing each nerve. All grids were scanned and the part of the perineurium lying midway between two grid bars was photographed together with the underlying part of the nerve. Areas where blood vessels appeared in the perineurium were avoided. The relative grain density over the outer, middle and inner third of the perineurium, as well as the grain density over the subperineurial part of the endoneurium was determined for each grid. The subperineurial part of the endoneurium was defined as the area between the inner limit of the perineurium and a line drawn 20 mm (2.8 μm) inside the perineurium but excluding areas occupied by myelin sheaths. The respective areas were calculated by point counting. The approximate background activity was obtained from control areas. These were defined as the endoneurial area of the micrographs, including nerve fibres and other structures at a distance of at least 5 microns from the inner limit of the perineurium. The study included altogether 24 grids and 296 autoradiograms. Of totally 2412 grains counted, 294 were located over the control areas, which were several times larger than the perineurial and subperineurial areas, indicating a low background activity.

Macrophages in normal peripheral nerves

In order to identify endoneurial macrophages, three characteristic but not specific features of macrophages were exploited. These were the high activity of certain enzymes (Braunsteiner and Schmalzl 1970), the capacity of macrophages to endocytose foreign material and the ultrastructural appearance.

Ultrastructure: The rats were perfused with a solution containing 2 % glutaraldehyde and 2 % paraformaldehyde in 0.05 M sodium phosphate buffer. Specimens including the sciatic nerves and tail nerves were dissected out, rinsed in buffer and postfixed in 1 % OsO_4. After embedding in Epon 812, ultrathin sections (50 – 60 nm) were cut on an LKB ultrotome. The sections were poststained with uranyl acetate and lead citrate.

Acid phosphatase: Monocytes and macrophages generally show high activity of esterase and acid phosphatase (Braunsteiner and Schmalzl 1970). As high esterase activity is found in several more structures in the peripheral nerves compared to acid phosphatase when applying enzyme histochemistry (*cf.* Thomas 1977), localization of acid phosphatase activity has been investigated in

the present study. The method described by Barka and Andersson (1962) was applied. Anderson and Song (1962) have evaluated this method and described the localization of acid phosphatase in the nervous system. According to these authors, fixation in 4 % formol-calcium resulted in the best preservation of enzyme activity. Fixation in glutaraldehyde may also preserve enzyme activity in general (*cf.* Hayat 1970). Holtzman and Novikoff (1965) used glutaraldehyde in a study on the activity of acid phosphatase in peripheral nerves after crush injury. In this study the material was fixed by perfusion with 2 % glutaraldehyde in 0.2 M sodium phosphate-buffer. This method of fixation resulted in good differentiation of the level of acid phosphatase activity between the Schwann cells and the endoneurial cells which are proposed to be macrophages. After fixation, the sciatic nerves were rinsed in sodium phosphate buffer, and 12 μm thick cryostate sections were mounted on chromium-gelatine-treated glass slides. The sections were incubated for one hour in a medium containing α-naphtyl phosphate and hexazonium pararosanilin at pH 6 (see Barka and Anderson 1962). To study possible non-enzymatic staining, sections were also incubated in hexazonium pararosanilin (without substrate), in substrate (without hexazonium pararosanilin) or in incubation medium with the addition of an enzyme inhibitor (NaF). These control incubations resulted in no visible reaction product.

Colloidal carbon: One important function of macrophages is their capacity to endocytose foreign material, either by pinocytosis or by phagocytosis (Silverstein *et al.* 1977). This capacity was exploited by studying the uptake of colloidal carbon (Günther Wagner C11/1321a). The advantages of this method were as follows: 1. previous reports on rapid uptake of colloidal carbon by macrophages (Carr 1968), 2. the electron density of the carbon particles which makes it possible to trace them ultrastructurally and 3. suitable particle size (average size 30 nm according to Cotran *et al.* 1967), which resulted in a rather diffuse spread within the endoneurium after local injection. To avoid haematogenous admixture of macrophages the injections were performed *in vitro* after the sciatic nerves had been removed and mounted on cork plates. After the injections of colloidal carbon the specimens were kept in Eagle's MEM at 38° C for 20 minutes or 2 hours. The specimens were immersion-fixed but otherwise treated the same way as described above under: "Ultrastructure".

RESULTS

Motor nerve conduction velocity (MCV) and nerve fibre diameter (paper I)

The determinations of MCV revealed no obvious differences between the NP and LP rats during the studied period from 5 to about 30 weeks of age. In both groups the MCV increased from about 15 m/s at 5 weeks of age to about 30 m/s at 15 weeks of age. After this age no further increase in MCV was recorded. Nor were there any marked differences in nerve fibre calibre spectra between the NP and LP rats either at 6 or at 15 weeks of age (Fig. 4). In fact, statistical comparison (Fisher's permutation test) revealed that there was a tendency towards larger mean nerve fibre diameter in the dorsal tail nerve of the LP rats at 6 weeks of age ($2p < 0.05$). However, the inverse relation was found at 15 weeks of age ($2p < 0.05$). The mean nerve fibre diameter of the NP rats therefore increased more during the development from 6 to 15 weeks of age than the LP rats [($2\ p < 0.01$); Willcoxon's ranking test].

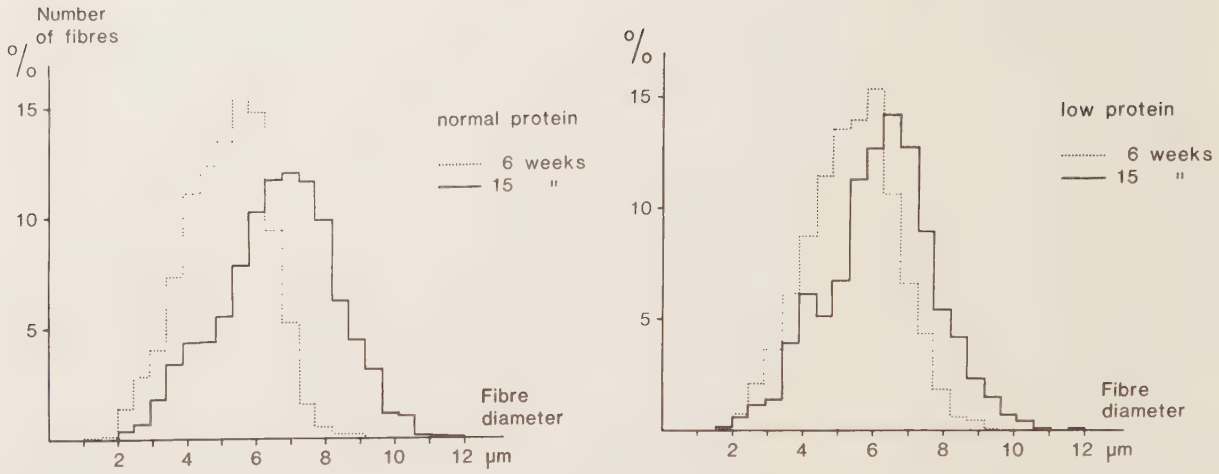

Fig. 4. Calibre spectra of myelinated nerve fibres of the dorsal tail nerve at 6 (– – – –) and 15 (——) weeks of age. Note the impaired increase of nerve fibre diameter in LP rats from 6 to 15 weeks of age.

Barrier studies:

Diphtheria toxin (paper II)

In all studied age-groups local application of DiT around the sciatic nerves resulted in a reversible hind limb paralysis (Fig. 5). The latency time from the day of toxin administration to the day of onset of paralysis increased with age. NP rats aged 26 weeks showed no signs of functional impairment until almost 5 weeks after toxin application. At 6 weeks of age the first signs of hind limb weakness appeared after about one week in the NP rats when the same dose of toxin was used. At 6, 12 and 26 weeks of age the course of functional disturbance was more protracted in the LP rats compared to the age-matched NP rats. At 6 and 12 weeks of age the LP rats were also more seriously affected than the NP rats in terms of degree of functional disturbance. At three weeks of age no obvious differences between the NP and LP rats were seen. The extent of nerve fibre damage was studied in three LP and three NP rats 16 days after toxin administration by means of nerve fibre teasing. The toxin had been applied at 12 weeks of age in these rats. The proportion of seriously damaged nerve fibres was found to be considerably larger in all these LP rats, which also showed a more marked functional disturbance than the NP rats at that time. When the degree of nerve fibre damage was compared between two LP and two NP rats (26 weeks of age) which showed equal signs of functional disturbance, no large differences between the groups were seen.

Horseradish peroxidase (HRP) (papers III and IV)

NP rats: In young rats (4 weeks of age) the tracer had diffused into the endoneurium within 2 hours(study III). It was then found between all perineurial laminae, in micropinocytotic vesicles of the perineurial cells, extracellularly in the endoneurium, in mesaxons of myelinated and unmyelinated nerve fibres and in the axons of some myelinated and unmyelinated nerve fibres. In rats aged 12 weeks (papers III and IV) no such extensive diffusion into the endoneurium was seen. At 10 and 30 minutes after the tracer application reaction product was seen only between the outer laminae of the perineurium and in micropinocytotic vesicles of the corresponding peri-

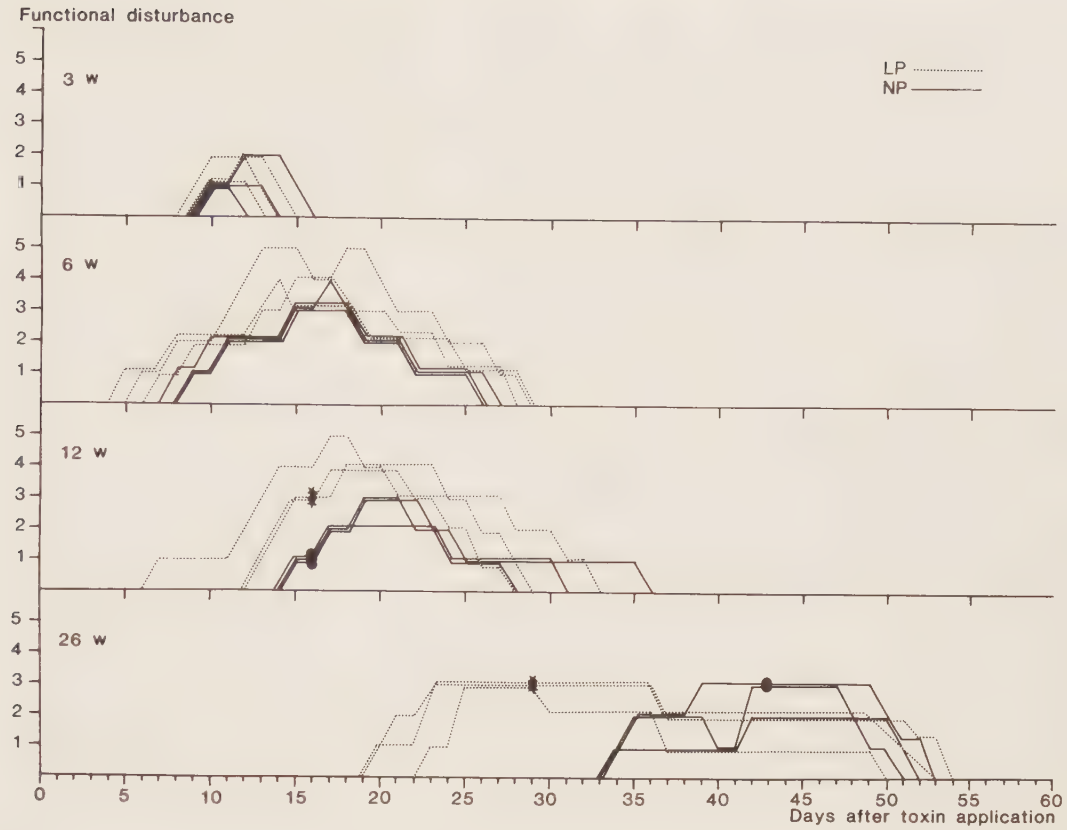

Fig. 5. Course of functional disturbance of LP (– – –) and NP (———) rats after local extraneural application of DiT at various ages. In rats aged 3 weeks the sciatic nerves were exposed to 0.5 Lf DiT. At 6, 12 and 26 weeks of age the nerves were exposed to 5 Lf. The degree of functional disturbance of each rat was estimated on a 6-point scale as proposed by Weller and Nester (1972): 1. Flattening of the foot and toes. 2. Adduction of the toes and weakness of the leg. 3. Inversion of the foot and crowding of the toes. 4. Flaccid paralysis of the foot with flexion of the toes. 5. Abduction and dragging of the foot. 6. Plantar hyperflexion of the ankle with the foot dragging behind.

neurial cells. Two hours after tracer application reaction product was identified deeper in the perineurium and also found in the cytoplasm and vacuoles of endoneurial cells, indicating passage of tracer into the endoneurium. Some of the perineurial micropinocytotic vesicles containing reaction product opened into extracellular spatia with no or only minor amounts of reaction product (Fig. 6). At 26 weeks of age the tracer distribution was similar to that found at 12 weeks of age. One day after tracer administration endoneurial reaction product was at all ages found only intracellularly within the endoneurium. Three days after the administration of HRP reaction product was found only occasionally and then intracellularly. In control nerves without HRP, endoneurial reaction product was never seen at the light microscopical level, except in red blood corpuscles.

LP rats: The distribution of reaction product differed between LP and NP rats at only one of the ages studied. In contrast to the NP rats, reaction product was found extracellularly within small nerve fascicles of LP rats at 12 weeks of age 2 hours after extraneural administration of HRP

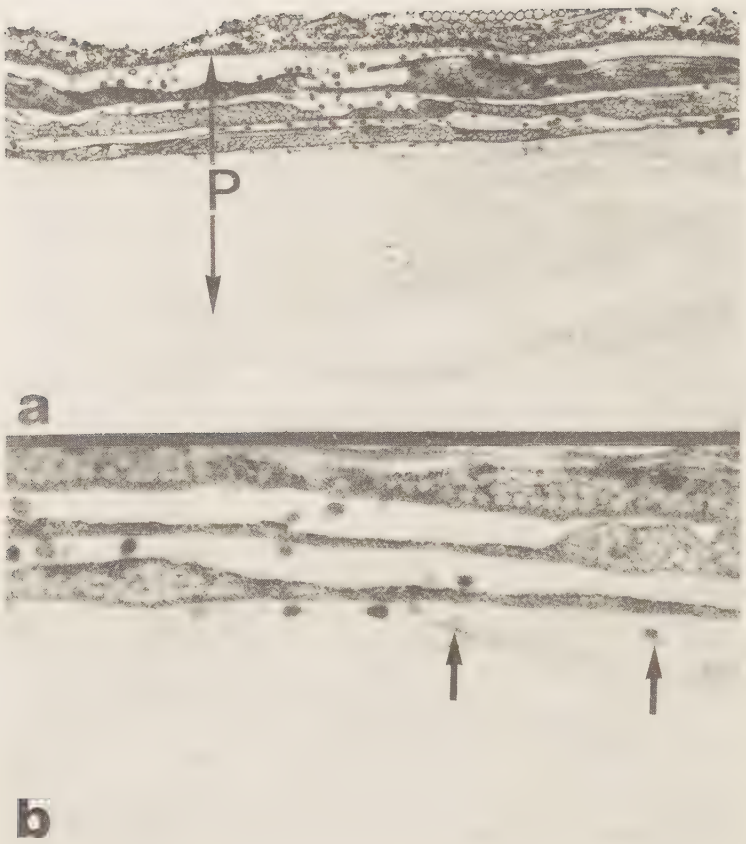

Fig. 6. Perineurium after extraneural application of HRP. a: Note multiple micropinocytotic vesicles filled with dark reaction product. b: Some vesicles containing reaction product seem to empty their content into intercellular spatia which contain minor amounts of reaction product (arrows). a: x 17.500; b: x 40.500.

(Fig. 7). This discrepancy was a constant finding in the five NP and three LP rats which were included in this part of the study.

^{125}I-labelled albumin (paper IV)

Part of one of the autoradiograms is illustrated in fig. 8.

The relative distribution of grains over the different parts of the perineurium 2 hours after local application of ^{125}I-labelled albumin in 2 LP and 2 NP rats is illustrated in fig. 9. The histograms are based on the number of counted grains per unit area after the adjustment for background activity.

The histograms show that 1. the number of grains decreases towards the inner part of the perineurium, 2. there is a specific activity over the inner third of the perineurium, 3. this activity is only about 1/10 of that over the outer third of the perineurium and 4. there are no obvious differences between the LP and NP rats. The specific activity over the subperineurial part of the endoneurium 2 hours after administration of the ^{125}I-labelled albumin was 2.6 – 4.9 per cent of the activity over the outer third of the perineurium. This activity was tested for specificity using Willcoxon's test for pair differences. In this test the activity of the control area was compared with

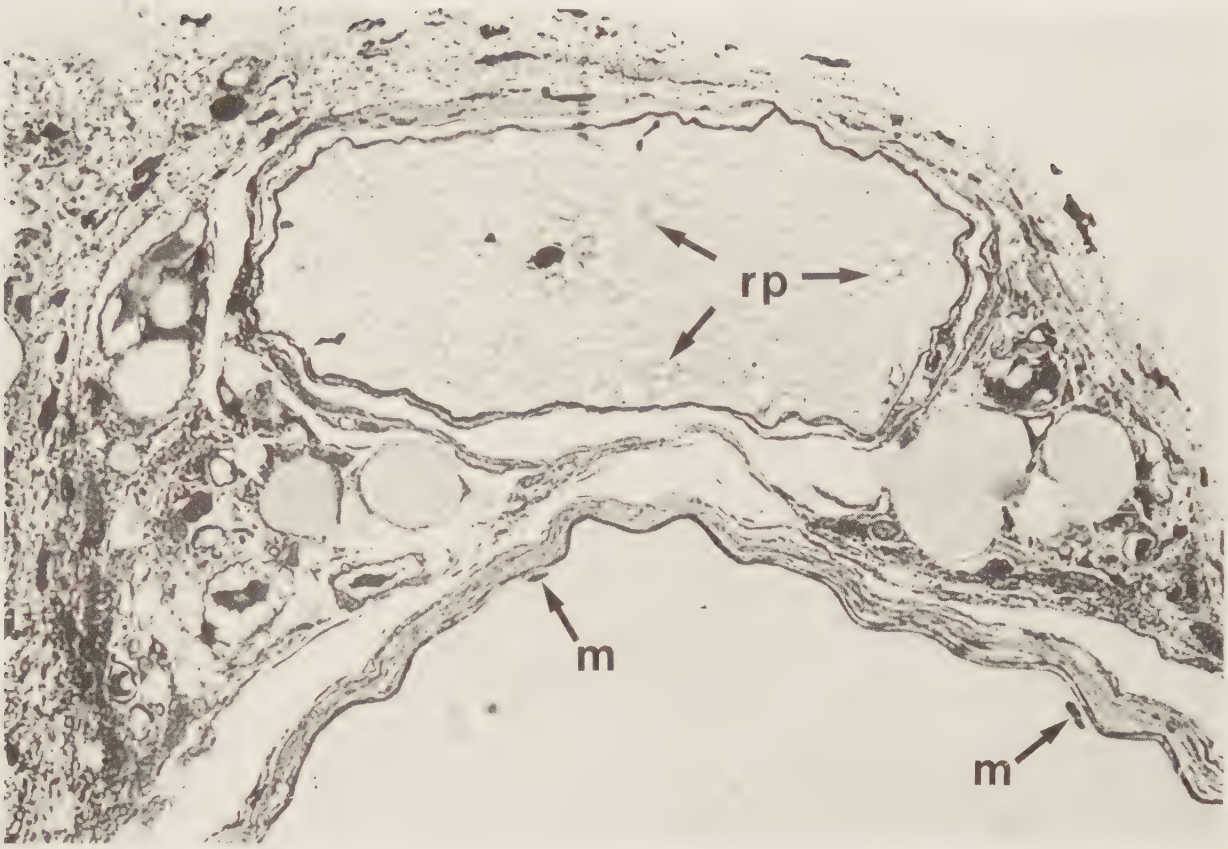

Fig. 7. Cross section of the sciatic nerve of a LP rat aged 12 weeks, 2 hours after extraneural application of HRP. The tracer has penetrated into a small nerve fascicle. The dark reaction product (rp) is seen between the nerve fibres in the endoneurium. In the large fascicle endoneurial reaction product is restricted to cells (m). x 450.

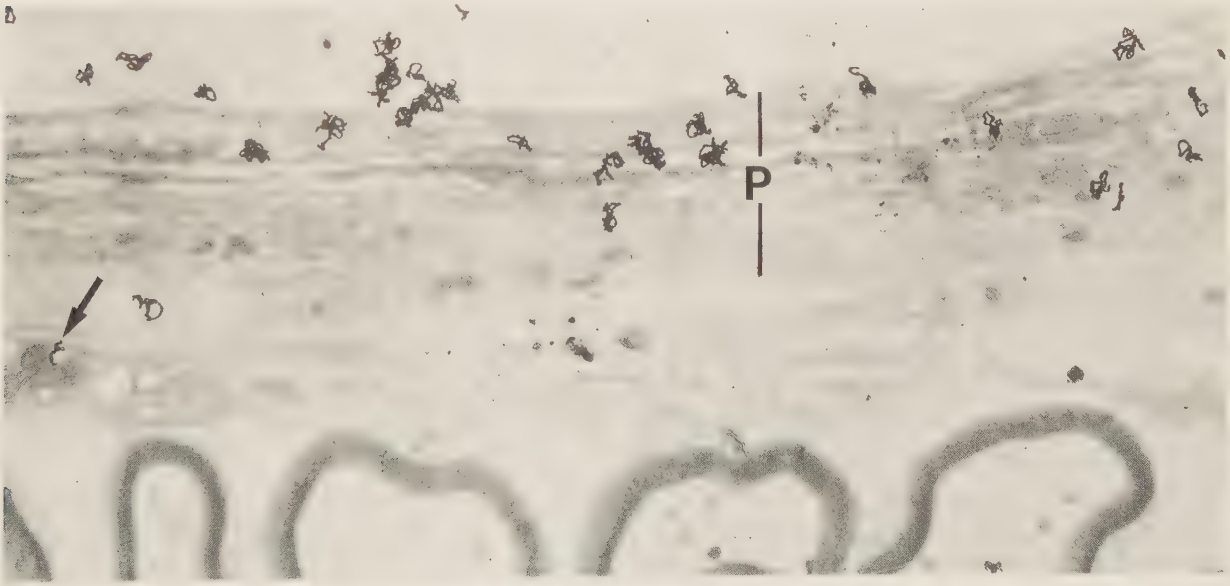

Fig. 8. Autoradiogram illustrating the perineurium (p) 2 hours after extraneural application of ^{125}I-labelled albumin. Autoradiographic grains are present over the epi-, peri- and endoneurium, as well as over an endoneurial cell (arrow). x 9.000.

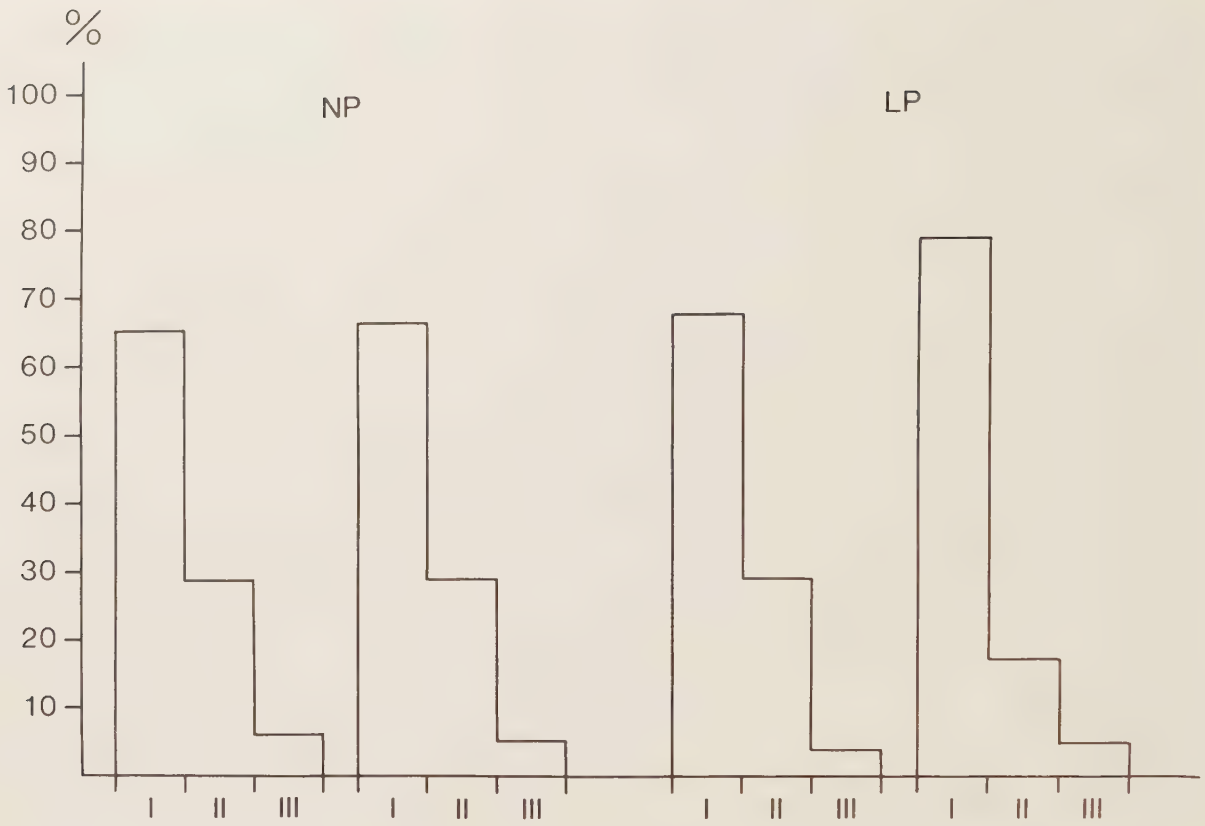

Fig. 9. Distribution of autoradiographic grains over the outer (I), middle (II) and inner (III) third of the perineurium 2 hours after extraneural application of ^{125}I-labelled albumin in 2 NP and 2 LP rats.

the activity over the subperineurial part of the endoneurium in the autoradiograms from each grid. In spite of the moderate number of grids, the activity of the subperineurial part of the endoneurium was significantly higher ($2p < 0.05$) than the activity over the control areas.

The distribution of grains over the outer, middle and inner third of the perineurium at 5 min. and 120 min. after the administration of ^{125}I-labelled albumin was compared using Pitman's test (Pitman 1937). The mean values of each group were used and the LP and NP rats were pooled. The test showed a statistically significant ($2p < 0.002$) shift of grain distribution to the inner parts of the perineurium with time. Although supporting the concept of an inward movement of the tracer with time, this does not necessarily mean that there was a passage of ^{125}I-labelled albumin from the outer to the inner parts of the perineurium during the period from 10 to 120 minutes. The two main reasons for this are 1. a diffusion or transport of ^{125}I-labelled albumin from the outer third of the perineurium in the opposite direction *i.e.* into the epineurium, would give the same result, and 2. the individual variation was not taken into consideration in this test.

Theoretically, the specific activity over the inner part of the perineurium and the subperineurial part of the endoneurium could be due to spread of radiation from the outer parts of the perineurium. Considering the relation between the thickness of the sections (< 0.09 μm) and the thick-

ness of the perineurium (ranging from 1 to 8 μm), this possibility is most unlikely (*cf.* Salpeter and Bachman 1972).

Macrophages in normal peripheral nerves (paper V)

Twenty minutes after endoneurial injection of colloidal carbon some endoneurial cells had accumulated carbon particles in large vacuoles. These cells had certain ultrastructural similarities to a type of cell found in the peripheral nerves of normal adult rats (Fig. 10). These cells lacked basement membranes and constituted 2 – 4 per cent of the endoneurial cell population. The main characteristic features which made it possible to differentiate them from endoneurial fibroblasts were 1. dark nucleus with cromatin condensed coarsely at the nuclear membrane, 2. numerous bristle-coated vesicles (> 100 nm), some of which were continous with the cytoplasmic membrane, 3. frequent dense bodies (lysosomes), 4. sparse rough endoplasmic reticulum and 5. sparsity of smooth micropinocytotic vesicles. These cells were located mainly in the vicinity of blood vessels and beneath the perineurium. Cells with high lightmicroscopical activity of acid phosphatase were distributed in the same way.

DISCUSSION

A. Normal animals

Diphtheria toxin (DiT) (paper II)

As the animals of all studied age-groups developed a hind limb paralysis, it is concluded that the peripheral nerve barriers to some extent were permeable to extraneurally applied DiT. In a previous study by Boettge (1975), using the same dose of toxin and the same mode of toxin application, no signs of neuropathy were seen up to 14 days after application of toxin in adult rats. This was misinterpreted as being due to the barrier function of the perineurium in adult rats. As has been shown in this study, and also previously by Jacobs (1967) after endoneurial toxin injections the latency time increases with age. In fact, the 26-weeks-old rats did not show any signs of functional disturbance until almost five weeks after toxin administration. The reason for this age difference is not clear but may be due to differences in the rate of myelin synthesis. The route of entrance of the toxin into the nerve could not be determined, but the previous concept of the perineurium as being impermeable to extraneurally applied macromolecules (see: Introduction) must be questioned. Theoretically, the toxin could also have passed into the endoneurium along blood vessels entering the endoneurium (Gunderson and Low 1968). The number of such sites has, however, been held to be of no importance for the barrier function (Burkel 1967). Another possibility is the uptake of toxin by epineurial vessels and entrance into the endoneurium *via* the vessels. Arvidsson (1977) has proposed such a mechanism as a possible explanation for the entrance of HRP from the epineurium into the endoneurium. A direct and rapidly damaging effect on the perineurium seems improbable as Boettge (1975) found no ultrastructural changes in the perineurium after DiT administration.

^{125}I-labelled albumin (paper IV)

The quantitative analysis of the autoradiograms has shown that 2 hours after the administration of ^{125}I-labelled albumin there was a specific activity over all parts of the perineurium as well as over the subperineurial part of the endoneurium. These findings must be interpreted as indicating

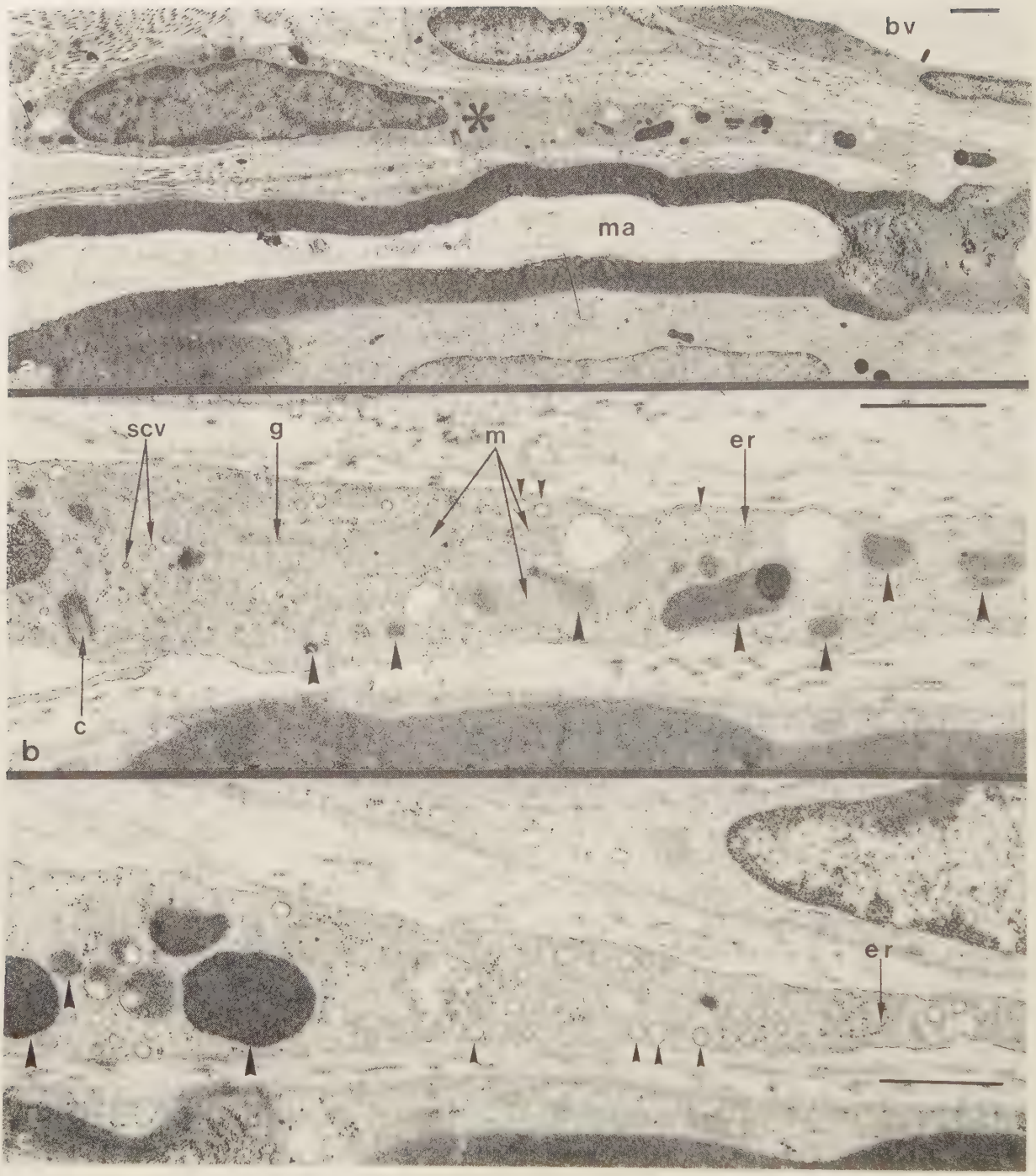

Fig. 10. Longitudinal section of a peripheral nerve in an adult rat. The elongated cell (*) in (a) shows numerous large bristle-coated vesicles (small arrow heads) and lysosomes (large arrow heads) (see b and c).
bv: blood vessel; ma: myelinated axon; m: mitochondria; g: Golgi apparatus; er: rough endoplasmic reticulum; c: centriole; scv: small coated vesicles. Bar corresponds to one μm.

a passage of the tracer across the perineurium. The results also show that this passage was limited. In previous studies on the perineurial barrier when fluorochrome-labelled albumin was used no passage into the endoneurium could be demonstrated in adult rats (Olsson 1966 a, Kristensson and Olsson 1971, Sima and Sourander 1973). The discrepancy between these studies and the present study may be interpreted as being due to the difference in sensitivity between the methods.

The result raises an important question: In what way do macromolecules pass the perineurium? Two possibilities may be considered: one is diffusion between the cells. However, zonulae occludentes are positioned at the cell contact of each perineurial layer. These are of an intermediate type *i.e.* ranging about midway between "very leaky" and "very tight" with respect to functional morphology according to Akert *et al.* (1976). The other possibility is that the passage is mediated by transport across the cells in micropinocytotic vesicles.

Horseradish peroxidase (HRP) (papers III and IV)

In young rats there was a diffuse leakage of HRP into the endoneurium. This is in accordance with the findings of Kristensson and Olsson (1971) and Sima and Sourander (1973, 1974). This leakage has been correlated to the morphology of the immature perineurium, which lack "tight junctions" between the perineurial cells (Kristensson and Olsson 1971). In previous studies in normal adult rats no endoneurial reaction product either extra- or intracellularly was described after the extraneural application of HRP (Klemm 1970, Böck and Hanak 1971, Sima and Sourander 1974, Kristensson and Olsson 1976). In this study, however, HRP was found to have accumulated in some endoneurial cells 2 hours after extraneural application of the tracer, indicating passage into the endoneurium. As was discussed with reference to DiT, this entrance into the endoneurium could have occurred at different sites. In the perineurium the tracer was identified between all except the innermost perineurial laminae. Furthermore, reaction product was found in numerous micropinocytotic vesicles of the perineurial cells. Some of these were intensely stained and obviously opened into intercellular spatia with minor amounts of reaction product (Fig. 6). Furthermore, the reaction product was found deeper in the perineurium after 2 hours exposure compared to the distribution after 10 minutes. The absence of unequivocally identified reaction product between the innermost layers of the perineurium and in the endoneurial extracellular space does not exclude the presence of HRP at these sites (see above under "Material and Methods"). The findings support the concept that vesicular transport of HRP across the perineurial cells may occur. Transcellular vesicular transport of protein tracers has also been demonstrated in endothelial cells (Karnovsky 1967, Bruns and Palade 1968, Simionescu *et al.* 1973, Johansson 1978 a, b). The presence of tracer-filled vesicles within the perineurial cells even remote from the cell surface must not, however, be taken as proof of vesicular transport (Olsson and Reese 1971). However, transport of HRP in micropinocytotic vesicles across the entire perineurium has been proposed to occur also in the newt after prolonged and repeated extraneural application of the tracer (Krishnan and Singer 1973).

Macrophages in normal peripheral nerves (paper V)

Doinikow (1911) described "resting wandering cells" or macrophages as belonging to the normal endoneurial cell population. However, in electron-microscopical studies on the cellular content of the peripheral nerves in the mouse, rat and rabbit (Causey and Barton 1959), the rabbit (Thomas 1963) and man (Ochoa and Mair 1969) no macrophages were encountered. It has been shown that macrophages occur in the spinal nerve roots of immature rats (Fraher and McDougall 1975)

and in spinal ganglia of adult rats (Jacobs *et al.* 1976) and mice (Arvidson 1979). In a study on barriers of the sciatic nerve in adult mice Arvidson (1977) noted endoneurial cells, which had endocytosed HRP. These cells were identified as active macrophages. In this study on the barriers of the sciatic nerve of rats we also found accumulation of HRP in endoneurial cells, presumably macrophages.

In study V a certain cell type with ultrastructural characteristics different from those of other known endoneurial cells was identified in normal peripheral nerves of rat. These characteristics, the capacity of these cells to endocytose colloidal carbon and the occurrence of endoneurial cells, with high activity of acid phosphatase, support the view that these cells are macrophages (Nichols *et al.* 1971, Carr 1973, Carr 1968, Braunsteiner and Schmalzl 1970). However, for the direct proof that these cells belong to the mononuclear phagocyte system (MPS) (van Furth *et al.* 1972, 1975) further experiments have to be carried out.

The normal occurrence of macrophages is interesting in the light of the debate concerning the origin of macrophages in different pathological conditions *e.g.* Wallerian degeneration (Olsson and Sjöstrand 1969, Asbury 1978, Berner *et al.* 1973, Oehmichen and Torvik 1976, Gibson 1979). It may be assumed that at least some of the active macrophages seen in this condition are derived from the normally occurring endoneurial macrophages.

Concluding remarks

The above-described and discussed properties of the perineurium as well as the occurrence of endoneurial macrophages give rise to some speculations. One is that the perineurium, besides acting as a protective diffusion barrier, also takes an active part in maintaining the homeostasis of the endoneurial environment by transport of substances not only in pathological conditions (Allt 1972, de la Motte *et al.* 1975), but also under normal conditions. This might be especially important in small nerve fascicles which lack endoneurial blood vessels, but also be of significance in the large nerve fascicles, which are held to lack lymphatic vessels (*cf.* Sunderland 1968). The perineurial area in relation to the number of enveloped nerve fibres is also considerably larger in small than in large fascicles. For further investigation of this subject it is important that small nerve branches covered with a single layer of perineurial cells are also studied. However, the structural organizaion of the perineurium of large nerve trunks with several layers of cells makes it an efficient barrier to local extraneural accumulation of noxious agents, although a limited vesicular transport across the cells may take place. As macrophages have the capacity of endocytosing foreign proteins like DiT (Mesrobeanu *et al.* 1966), the endoneurial macrophages may be considered to be an additional factor in a protective system of the nerve trunks. When a long segment of the nerve is exposed to a noxious agent like DiT this protective system obviously is not capable of preventing the damaging effect, however.

B. Protein deprived rats

Motor nerve conduction velocity (MCV) and nerve fibre diameter (paper I)

From the results obtained in the study on MCV (study I) it is concluded that the protein deprivation did not significantly influence this index of nerve fibre development. Nerve conduction velocity is positively correlated to the nerve fibre diameter (Hursh 1939). Accordingly there were no large differences of nerve fibre calibre spectra between LP and NP rats at 6 and 15 weeks of age. These results were obtained in spite of the considerably reduced body weight and the moderately

reduced levels of proteins in serum of the protein-deprived rats. At six weeks of age there was in fact a tendency to larger mean nerve fibre diameter in the protein-deprived rats. This can possibly be explained in the following way: During the first postnatal weeks, when the body growth of the LP rats was very limited the NP rats showed a rapid growth spurt. In a situation of rapid longitudinal growth myelin will be formed around rapidly elongating axons. This may influence the increase in fibre diameter. At 15 weeks of age the NP rats showed somewhat larger mean nerve fibre diameter than the LP rats, however. Thus the protein-deprived rats showed a decreased nerve fibre calibre growth during the period from 6 to 15 weeks of age. During this period the increase per day in body weight and body length of the LP rats was larger than in the period from 0 to 6 weeks of age. The results thus indicate that the nerve fibre calibre growth and increase of MCV do not run strictly parallel to the increase of body length and weight.

At 15 weeks of age the LP rats showed slightly decreased mean nerve fibre diameter, however. In previous studies on the effects of undernutrition on the peripheral nerve fibres, the reduction of nerve fibre diameter has been shown to be an effect of both decreased growth of axonal circumference and impaired myelin formation (Hedley-Whyte and Meuser 1971, Hedley-Whyte 1973, Sima 1974 b). In a study on the conduction velocity of the fastest fibres of the dorsal roots, Sima and Jankowska (1975) showed a small reduction of conduction velocity in pre- and post-natally undernourished rats aged 180 days. This reduction was proportional to the reduced diameter of the corresponding nerve fibres. Considering the method used in this study on MCV in protein deprived rats, minor differences between the nutritional groups could not be detected. This depends on the facts that not only the fastest fibres were included, but a population of different motor fibres, and that the determinations of MCV were performed on rats at various ages.

Diphtheria toxin (DiT) (paper II)

Differences in the effect of the extraneurally applied DiT between the LP and NP rats were manifested at 6, 12 and 26 weeks of age but not at 3 weeks of age. The differences noted were as follows: 1. a shorter latency time in LP rats, 2. a protracted course of functional disturbance in LP rats and 3. a higher degree of maximal functional disturbance in LP rats. All these differences between the nutritional groups were a constant finding at 6 and 12 weeks of age. These variables are correlated to the dose of toxin after endoneurial injections (Cavanagh and Jacobs 1964, Jacobs 1967). A possible explanation for the differences noted might therefore be less efficient protective barriers in the LP rats at 6 and 12 weeks of age. However, the differences in latency time might possibly be explained by differences in the rate of myelin synthesis. The protracted course of functional disturbance could be explained by a reduced reparative capacity of the Schwann cells in LP rats. The higher degree of functional disturbance noted in the LP rats might possibly be due to greater vulnerability of the Schwann cells.

Horseradish peroxidase (HRP) (paper III)

The differences between LP and NP rats were restricted to one age-group: rats aged 12 weeks. At this age a persistence of extensive leakage of HRP into the endoneurium was noted in the LP rats in small fascicles of the sciatic nerve. This finding is interpreted as indicating a delayed maturation of the perineurium as a diffusion barrier in LP rats. Whether this leakage was due to incomplete zonulae occludentes between the perineurial cells or vesicular transport across the perineurial cells could not be established

^{125}I-labelled albumin (paper IV)

The study included the large fascicle constituting the proximal part of the sciatic nerve. No constant differences in the distribution of grains over the perineurium and endoneurium in LP and NP rats were found. The results do not exclude the existence of minor differences in the permeability of the perineurium. Such possible differences must, however, be studied with statistical methods and in larger groups of animals.

Concluding remarks

The delayed maturation of the perineurium as a diffusion barrier as showed with HRP probably contributed to the increased vulnerability noted in LP rats after extraneurally applied DiT at 6 and 12 weeks of age. The delayed development of the perineurium is in accordance with the previous reported delayed development of enzyme activities in the perineurium of sciatic nerve in undernourished rats (Thomas and Sourander 1977). It has previously been claimed that pre- and postnatal undernutrition may cause impaired function of the perineurium as a diffusion barrier to macromolecules even in older rats (Sima and Sourander 1973, 1974). In the present investigation it has been shown that the perineurium to some extent is permeable to macromolecules even in normal adult rats. Although the permeability of the perineurium in small fascicles of the sciatic nerve differed between LP and NP rats at 12 weeks of age in this study, such a marked difference did not persist to 26 weeks of age.

The stage of development, at which the perineurium appears with its mature structure and function, shows interspecies variations. In contrast to mice and rats, the barrier function of the perineurium is fully developed in newborn guinea pigs (Kristensson and Olsson 1971). The age at which the maturation of the perineurium appears in man is not known, but according to own observations, the perineurium of large nerves acts as a diffusion barrier to macromolecules at the 28th gestational week. The perineurium appeared morphologically immature in a foetus aged 14 weeks (Gamble and Breathnach 1965).

As endoneurial macrophages may constitute a part of a protective system of the peripheral nerves, the influence of protein deprivation on macrophages is of interest. It has been shown by Guggenheim and Buechler (1948, 1949) that protein deprivation in rats may impair regeneration and phagocytic activity of leucocytes. However, with respect to cellular morphology and IgG receptors, rat macrophages remained normal after severe protein deprivation (Keusch *et al.* 1978).

The increase of nerve fibre diameter during development was slightly influenced by the protein deprivation. This influence could partly be explained by the altered growth pattern of the LP rats. While the growth spurt both in terms of body weight and body length was considerably delayed in the LP rats, the increase of MCV in LP rats followed essentially the same curve as that of the NP rats. From these results it may be concluded that the nerve fibre development in terms of calibre growth and increase of MCV is primarily correlated to chronological age.

Extreme reduction of MCV, which has been recorded in children suffering from PCM (Osuntokun 1971, Sachdev *et al.* 1971, Engsner and Woldemariam 1974, Singh *et al.* 1976) has not been reproduced in rats. This marked reduction of MCV was however restricted to severe cases of PCM. MCV was normalized within a few weeks of nutritional rehabilitation. Thus it seems improbable that this extreme reduction of MCV was an effect of reduced nerve fibre calibres. Several other mechanisms may have contributed to the observed reduction of MCV, as nerve conduction velocity

is dependant on various factors *e.g.* extracellular sodium concentration (Hodgkin and Katz 1949) and temperature (Paintal 1965). Platt and Stewart (1969) have reported paranodal myelin retraction in dogs fed a low protein diet.

Roy *et al.* (1972) reported ultrastructural alterations in the myelin sheaths of nerve fibres in monkeys fed a low-protein diet. An experimental model of kwashioskor in rats, implying severe protein deprivation, has been described by Edozien (1968). So far, no studies on the peripheral nervous system in such rats have been published, however.

Considering the very limited alterations of the nerve fibre calibre growth in the LP rats the findings reported by Morgane *et al.* (1978) are of interest. They have shown that the increase of brain weight in relation to body weight is larger in protein-deprived rats than controls during the first few postnatal weeks, suggesting a brain-sparing phenomenon.

When discussing the effects of PCM in rats the dwarfism induced by nutritional deprivation must be taken into consideration. Decreased body weight gain resulting in body weights of only 1/12 of age-matched controls has been produced in rats by underfeeding (Haltia 1970). Such extreme dwarfism is not seen in man after nutritional deprivation. It is therefore important to determine the content of albumin in serum to obtain information on the nutritional state of the rats. In this study the content of albumin and total protein in serum suggested a moderate protein deprivation, compared to that described by Edozien (1968).

SUMMARY AND CONCLUSIONS

The study may be summarized as follows:

1. Rats were protein-deprived by feeding female rats a protein-deficient diet, which contained 7 weight per cent protein, before, during and after pregnancy. After weaning the offspring were fed the same protein-deficient diet. Control rats were given a diet containing 14 weight per cent protein. The protein deprivation resulted in considerably retarded body growth and moderately reduced values of albumin and total protein in serum.

2. The development of the peripheral nerve fibres was evaluated in morphometric and functional studies of the dorsal tail nerve. These revealed that the motor nerve conduction velocity was not significantly affected by the protein deprivation during the development from 5 to 30 weeks of age, and that the mean nerve fibre diameter was slightly altered at 6 and 15 weeks of age.

3. Diphtheria toxin, which is known to cause segmental demyelination after endoneurial injection was locally applied around the sciatic nerves of normal and protein-deprived rats at different ages. Diphtheria toxin penetrated into the endoneurium of both protein-deprived and normal rats at all ages. This resulted in nerve fibre damage with concomitant reversible hind limb paralysis. The results clearly indicated that the protein-deprived rats were more vulnerable at 6 and 12 weeks of age. Even in rats aged 26 weeks the course of functional impairment was more protracted in protein-deprived rats.

4. Horseradish peroxidase (HRP) readily diffused into the sciatic nerves after extraneural application in young but not in adult rats. This finding was in accordance with previous reports of differences in permeability of the perineurium during development. It was also found, how-

ever, that the tracer accumulated in endoneurial cells even in adult rats indicating penetration of the tracer into the endoneurium. The distribution of tracer in the perineurium supported the concept of a transcellular vesicular transport of horseradish peroxidase across the perineurial cells.

The maturation of the perineurium as a diffusion barrier to HRP was delayed in protein-depreived rats, indicating by a remaining leakage of HRP into small nerve fascicles of the sciatic nerve at 12 weeks of age.

5. The passage of ^{125}I-labelled albumin across the perineurium of the proximal part of the sciatic nerve was studied with ultrastructural autoradiography in normal and protein-deprived rats aged 12 weeks. Passage of this tracer was demonstrated by the presence of autoradiographic grains over all layers of the perineurium as well as over the subperineurial part of the endoneurium 2 hours after the extraneural application of ^{125}I-labelled albumin. This penetration was a limited process, however, as indicated by the considerably lower activity over the inner part of the perineurium compared to the outer part. No obvious differences were noted between protein-deprived and normal rats.

6. In view of the observed accumulation of HRP in endoneurial cells, a study was made on the occurrence of macrophages in normal peripheral nerves. Endoneurial cells with ultrastructural characteristics of macrophages were identified. These cells which rapidly endocytosed colloidal carbon *in vitro*, were located mainly in the vicinity of blood vessels or near the perineurium. High activity of acid phosphatase was found light-microscopically in cells with the same distribution.

Conclusions: The mature perineurium of rat sciatic nerve is to some extent permeable to macromolecules. The study supports the concept of trans-cellular vesicular transport in perineurial cells. The significance of such a vesicular transport for the maintainance of normal endoneurial homeostasis deserves further study. Cells exhibiting features characteristic of macrophages belong to the normal endoneurial cell population. These cells may contribute to a protective system of the peripheral nerves. Pre- and postnatally protein-deprived rats show a considerably delayed and decreased body growth, while the increase in nerve fibre diameter is influenced to a minor degree. The motor nerve conduction velocity and nerve fibre diameter are correlated to chronological age and not to body size during the postnatal development. Pre- and postnatal protein deprivation results in delayed maturation of the perineurium as a diffusion barrier in the sciatic nerve, which leads to increased vulnerability to extraneurally applied diphtheria toxin.

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